

Vibriocidal antibodies induced by *Yersinia enterocolitica* serotype IX

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SUMMARY

Yersinia enterocolitica serotype IX has been found to stimulate the production of vibriocidal antibody against *Vibrio cholerae*, particularly in Inaba serotype, in experimental rabbits and infected man to a significant degree. This activity could be absorbed by both Inaba and *Yersinia* antigens from anti-yersinia sera, but *Yersinia* antigen could not absorb vibriocidal activity from the anti-cholera sera, indicating a unilateral relation.

Vibrio agglutinating antibody, particularly against Ogawa, in anti-yersinia rabbit or human sera was found to be less liable to non-specific stimulation.

V. cholerae, while removing vibriocidal antibody, could not absorb the *Yersinia* agglutinin from anti-yersinia sera, suggesting that there is a different determinant for these two types of antibody activities.

The non-specific production of vibriocidal antibody by *Y. enterocolitica* type IX indicates the need for caution in the interpretation of the results of vibriocidal tests where such organisms are prevalent.

INTRODUCTION

Yersinia enterocolitica is not a very well known organism and has only recently been differentiated from *Pasteurella pseudotuberculosis* on the basis of biochemical and serological tests. It was referred to as *Pasteurella X* by Knapp & Thal (1963) and Carlsson, Ryd & Sternby (1964), but Frederiksen (1964) proposed the designation of *Y. enterocolitica*. It is known to occur in animals such as chinchillas, hares, pigs and dogs, and has also been suspected of causing terminal ileitis, mesenteric lymphadenitis (clinically simulating acute appendicitis), diarrhoea and erythema nodosum in man (Winblad, Niléhn & Sternby, 1966; Niléhn, 1967; Ahvonen & Sievers, 1969). In 1967, Winblad described eight serotypes of *Y. enterocolitica* based on O antigens, but his collection of 105 strains did not include the serotype which cross-reacts strongly with *Brucella* species and which was described by Ahvonen & Sievers (1969) as *Y. enterocolitica* serotype IX.

Tests for vibriocidal and agglutinating antibodies have been increasingly employed in recent years in studies on the immunology and epidemiology of

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cholera. The presence of vibriocidal antibody in the sera of American citizens who have never been vaccinated against cholera nor exposed to cholera infection (Finkelstein, Powell, Woodrow & Krevans, 1965; Verwey *et al.* 1969) has raised the question of the specificity of the vibriocidal reaction. Cross-reactivity between *Vibrio cholerae* and *Brucella* species is widely known (Eisele, McCullough, Beal & Burrows, 1946; Gallut, 1950; Mathur, 1960) and has recently been again emphasized by Feeley (1969) and Gangarosa, DeWitt, Feeley & Adams (1970), who found other instances of the presence of vibriocidal antibody in human sera in the absence of exposure to cholera infection. Corbel & Cullen (1970) have also recently demonstrated cross-reacting antibodies induced by *Br. abortus* and *Y. enterocolitica* serotype IX in cattle by various techniques.

Infection by *Y. enterocolitica* appears to be fairly common in countries where attempts are made to look for the organism. Recently Makulu, Gatti, Mollaret & Vandepitte (1969) reported 11 isolations in 8 months in the Democratic Republic of the Congo.

This investigation was undertaken to elucidate the possible antigenic relationship between *V. cholerae* and *Y. enterocolitica* serotype IX which may have an influence on sero-epidemiological studies.

METHODS AND MATERIALS

Strains and sera

Strains of *V. cholerae*, Ogawa NIH 41 and Inaba V 86, from the lyophilized stock culture in this laboratory were used to prepare antigens for the immunization of animals as well as for antibody titrations.

Strain M.Y. 79 of *Y. enterocolitica* serotype IX and nine human sera from bacteriologically confirmed cases of infection by this organism were kindly provided by Dr P. Ahvonen, Helsinki, Finland. In this communication the term '*Yersinia*' refers to this particular serotype.

Immune sera against *V. cholerae* Ogawa and Inaba were prepared in rabbits (four for each serotype) by injecting live saline suspensions from a 7 hr.-old growth (at 37° C.) on heart infusion agar containing about 2×10^9 organisms per ml. Each of the rabbits received three intravenous injections, 5 days apart, of 0.25, 0.5, and 0.5 ml.; they were bled seven days after the last injection and the sera were pooled as Ogawa and Inaba.

Four rabbits were used to prepare antisera against *Y. enterocolitica* serotype IX. Two of them received two intravenous injections, seven days apart, of 0.25 and 0.5 ml. of live saline suspension from a 48 hr.-old growth (at about 22° C.) on heart infusion agar containing about 2×10^9 organisms per ml. Identical suspensions were heated at 120° C. for two hours and similarly injected into two other rabbits. Blood was collected 7 days after the second injection. Sera against the live and heated antigens were pooled separately and all the sera were stored at -20° C.

Agglutination test

Heated antigen was preferred to live antigen as this permitted repetition of the tests using the same antigen. Saline suspensions from a 4 hr.-old growth of *V. cholerae* on heart infusion agar at 37° C. were heated at 100° C. for 1 hr. for the preparation of Ogawa and Inaba antigens. Forty-eight-hour-old growth of *Y. enterocolitica* (as it grows slowly) on heart infusion agar at about 22° C. was suspended in saline and heated at 100° C. for 2 hr. for the preparation of *Yersinia* antigen. These suspensions were washed once in saline and then adjusted to Klett-Summerson colorimeter reading 30 (OD 0.061) using a green filter.

Equal volumes of the antigens were added to 0.5 ml. volumes of the serial 2-fold dilutions of the sera in normal saline in round-bottomed 10 × 90 mm. tubes and then placed in a 52° C. water bath overnight.

Tests were read with the naked eye; the highest dilutions showing fine but definite clumps and clearing of the suspending medium constituted the end-point.

Antisera were also tested in the same way with saline suspensions of live *V. cholerae* Inaba and Ogawa and *Yersinia* grown on heart infusion agar after standardizing the suspensions to the same optical density.

Vibriocidal test

The test was done as previously described by Verwey *et al.* (1969). Serial 2-fold dilutions of the sera in Kolmer's saline in 0.2 ml. volumes were mixed with equal volumes of the complement-cell mixture (complement 1/10 (Hyland Lab.); *V. cholerae* Inaba or Ogawa, 2×10^8 per ml.) and incubated in a 37° C. water bath for 1 hr. with occasional shaking. Brain heart infusion broth, 1.6 ml., was then added to all tubes which were incubated again for 2–2½ hr. The tubes were then examined with the naked eye for the amount of growth of *V. cholerae* as compared with that in the control tube; the degree of turbidity was graded from 0 to 4+, and a 2+ growth was considered as the 50% end-point. Appropriate controls and reference sera were included in each test.

A Brewer machine was used for distribution of the vehicle and for adding broth, and an automatic serum diluter (American Instrument Co.) was used for serum dilution.

The results of the agglutinating and vibriocidal tests are expressed in reciprocals of the titres.

Absorption of the sera

The antigens were prepared in the same way as for the agglutination test, but the suspension contained 1×10^{11} organisms per ml. In the first stage of absorption, 0.5 ml. of the antigen was diluted with 5 ml. of saline and centrifuged at 12,100 *g* for 30 min. The bacterial deposit was carefully mixed with 5 ml. of a 1/5 dilution of the serum to be absorbed. The mixture was incubated at 37° C. in a water bath for 2 hr. with occasional shaking, and then centrifuged at 26,384 *g* for 30 min. For the second absorption, this supernatant was mixed with the bacterial mass from 2.5 ml. of antigen suspension diluted with 5 ml. of saline and centrifuged at

12,100 *g* for 30 min. The mixture was incubated again in a 37° C. water bath for 2 hr. with occasional shaking and then kept at 4° C. overnight before being centrifuged at 26,384 *g* for 30 min. The supernatant was collected and stored at -20° C.

RESULTS

Y. enterocolitica serotype IX strain M.Y.79 did not react well in the complement-dependent bactericidal system with the homologous or anti-*V. cholerae* sera. In the agglutination test the heated O antigen of this organism was found to react better than live suspension; antisera prepared by injecting live or heated *Yersinia* did not produce any visible clumping with live homologous antigen, as shown in Table 1. This table also shows that anti-cholera sera agglutinated heated *Yersinia* antigen to a titre of only 40-80 and did not react at all with *Yersinia* live antigen. Anti-yersinia sera, on the other hand, exhibited very high vibriocidal activity against Inaba serotype; two out of the four rabbits, one receiving live and another heated antigen, also developed Ogawa vibriocidal titre to a moderate degree. One of the four anti-yersinia sera revealed some agglutinin titre against heated Inaba and Ogawa antigen; all of them had some agglutinating activity against live Inaba (OH) antigen.

The results after two-step absorption of the anti-cholera and anti-yersinia sera with heated *V. cholerae* (Ogawa and Inaba) and *Yersinia* antigen are shown in Table 2. The titres of the agglutination test appear to be different in this table from those in Table 1, because the initial dilution of the sera in this case was 1/15. Table 2 shows that the group-specific vibriocidal and agglutinating activity of anti-cholera sera could be removed by absorbing the anti-Inaba serum with Ogawa antigen and anti-Ogawa serum with Inaba antigen in appropriate quantities

Table 1. *Vibriocidal and agglutinating antibody titre of immune rabbit sera*

	Vibriocidin titre		Agglutinin titre					
			Inaba		Ogawa		<i>Y. ent.</i> (IX)	
	Inaba	Ogawa	Live	Heated	Live	Heated	Live	Heated
<i>V. cholerae</i>								
Inaba-Live (K)	163,840	163,840	2,560	2,560	2,560	1,280	—	80
<i>V. cholerae</i>								
Ogawa-Live (J)	327,680	327,680	1,280	1,280	5,120	1,280	—	40
<i>Y. enterocolitica</i>								
Serotype IX	10,240	1,280	40	—	—	—	—	10,240
Live (75)								
Live (73)	2,560	20	40	—	—	—	—	2,560
<i>Y. enterocolitica</i>								
Serotype IX								
Heated (77)	40,960	20	160	—	—	—	—	2,560
Heated (80)	5,120	1,280	40	20	—	80	—	2,560

Pre-immunization vibriocidal titres against Ogawa and Inaba were less than five in all animals except Nos. 80 and 75 in which the titres against Inaba were ten and five respectively.

‘—’ = less than 10.

but not by the *Yersinia* antigen. Inaba vibriocidal activity in the anti-yersinia sera could be removed by Inaba antigen but not by Ogawa, and neither of the two vibrio antigens could reduce homologous *Yersinia* agglutinin titre. Ogawa vibrio-

Table 2. *Antibody titre of immune rabbit sera before and after absorption with heated homologous and heterologous antigens*

(Agglutinating titre with heated antigens only)

Sera	Absorbed with	Vibriocidin titre		Agglutinin titre		
		Inaba	Ogawa	Inaba	Ogawa	<i>Y. ent.</i> (IX)
<i>V. cholerae</i>	—	163,840	163,840	3,840	3,840	60
Inaba (K)	Inaba	20	—	—	—	—
	Ogawa	81,920	1,280	1,920	—	—
	<i>Y. ent.</i> (IX)	163,840	163,840	1,920	1,920	—
<i>V. cholerae</i>	—	327,680	327,680	960	1,920	60
Ogawa (J)	Inaba	40	81,920	—	1,920	—
	Ogawa	—	40	—	—	—
	<i>Y. ent.</i> (IX)	163,840	163,840	960	960	—
<i>Y. ent.</i> (IX)	—	40,960	20	—	—	—
Heated (77)	Inaba	—	—	—	—	3,840
	Ogawa	20,480	—	—	—	3,840
	<i>Y. ent.</i> (IX)	—	—	—	—	3,840
<i>Y. ent.</i> (IX)	—	10,240	1,280	—	—	7,680
Live (75)	Inaba	—	40	—	—	3,840
	Ogawa	10,240	—	—	—	7,680
	<i>Y. ent.</i> (IX)	—	—	—	—	—

Vibriocidin titre: '—' = less than 10.

Agglutinin titre: '—' = less than 30.

Table 3. *Antibody titre of immune rabbit sera before and after absorption of anti-V. cholerae sera with live Y. enterocolitica antigen*

(Agglutinating titre with heated antigens only)

Sera*	Absorbed with	Vibriocidin titre		Agglutinin titre		
		Inaba	Ogawa	Inaba	Ogawa	<i>Y. ent.</i> (IX)
Inaba (K)	—	163,840	163,840	2,560	1,280	80
Inaba (K)	<i>Y. ent.</i>	163,840	81,920	2,560	1,280	80
Ogawa (J)	—	327,680	327,680	1,280	1,280	40
Ogawa (J)	<i>Y. ent.</i>	327,680	327,680	1,280	1,280	40
Monovalent Inaba	—	2,560	80	40	—	—
Monovalent Inaba	<i>Y. ent.</i>	2,560	80	40	—	—
Monovalent Ogawa	—	80	5,120	—	160	—
Monovalent Ogawa	<i>Y. ent.</i>	80	5,120	—	160	—

* Inaba (K) and Ogawa (J) sera were unabsorbed anti-cholera sera containing both group and type antibodies.

Monovalent Inaba and Ogawa sera were absorbed by heterologous antigens and, therefore, type-specific.

'—' = less than 10.

cidal titre seen in one anti-yersinia serum was absorbed by Ogawa antigen. Absorption with *Yersinia* antigen of the homologous sera removed both the heterologous vibriocidal and the homologous agglutinating activity almost completely.

Table 3 shows that the live *Yersinia* antigen, like the heated antigen, could not absorb the vibriocidal and agglutinating antibodies of polyvalent anti-cholera sera (with both group and type-specific antibodies) nor those of monovalent Inaba and Ogawa sera (with type-specific antibody only) kindly supplied by Dr H. Smith of the U.S. Vibrio Reference Center.

Table 4. *Vibriocidal and agglutinating activities of sera from patients with infection by Y. enterocolitica serotype IX*

	Vibriocidin titre		Agglutinin titre (heated antigen)		
	Inaba	Ogawa	Inaba	Ogawa	<i>Y. ent.</i>
1 *(2215)	—	—	—	—	—
2 (3208)	640	320	160	—	320
3 (1287)	10,240	160	160	—	160
4 (954)	20	80	—	—	640
5 (112)	320	40	40	40	320
6 (3649)	1,280	640	40	—	320
7 (4381)	160	40	—	—	160
8 (2207)	20	—	—	—	40
9 (1246)	5,120	40	—	—	80
10 (1159)	5,120	20	—	—	1,280

* Serum from a patient infected with *Y. enterocolitica* of a different serotype.
'—' = less than 20.

Table 5. *Vibriocidal and agglutinating antibody titre of sera from patients with infection by Y. enterocolitica serotype IX*

(After homologous and heterologous absorption with heated antigen.)

Sera	Absorbed with	Vibriocidin titre		Agglutinin titre		
		Inaba	Ogawa	Inaba	Ogawa	<i>Y. ent.</i>
2	—	640	320	160	—	320
	<i>Yersinia</i>	40	—	—	ND	—
	Inaba	—	—	—	ND	320
3	—	10,240	160	160	—	160
	<i>Yersinia</i>	—	—	—	—	—
	Inaba	—	—	—	ND	160
6	—	1,280	640	40	—	320
	<i>Yersinia</i>	—	—	ND	ND	ND
9	—	5,120	40	—	—	80
	<i>Yersinia</i>	—	—	—	ND	—
	Inaba	—	—	—	ND	80
10	—	5,120	20	—	—	1,280
	<i>Yersinia</i>	—	—	—	ND	—

'ND' = not done.

'—' = less than 20.

The results of the vibriocidal and agglutination tests on ten human sera are shown in Table 4; nine of them were from bacteriologically confirmed cases of *Y. enterocolitica* serotype IX. Serum No. 2215 from a patient with infection by *Y. enterocolitica* of a different serotype did not show any demonstrable antibody against *Y. enterocolitica* serotype IX or *V. cholerae* in tests employed. Of the nine other sera, all of which had *Yersinia* agglutinin titres varying from 40 to 1280, seven had high vibriocidal activity against Inaba; the Ogawa vibriocidal titre was less pronounced. Agglutinin titre with *V. cholerae* was not remarkable in most of the sera, particularly against Ogawa.

The absorption of five of these sera with heated antigens of Inaba and *Yersinia* again revealed that *V. cholerae* Inaba antigen could remove the vibriocidal activity but not the *Yersinia* agglutinating activity whereas *Yersinia* antigen could absorb both the activities (Table 5).

DISCUSSION

The results of this study show that *Y. enterocolitica* serotype IX induces the production of vibriocidal antibody, particularly against serotype Inaba, both in the rabbit and in man. This was not unexpected in view of the previous reports on cross agglutination between *Y. enterocolitica* serotype IX and *Brucella* species (Ahvonen & Sievers, 1969; Ahvonen, Jansson & Aho, 1969) and also between *Brucella* species and *V. cholerae* (Feeley, 1969; Gangarosa *et al.* 1970). However, in the present study, the antigenic relationship between *Yersinia* and *V. cholerae* appeared to be rather complex.

It was not possible to perform classical absorption studies of the antisera for the determination of antigenic relationship between the relevant organisms for the following reasons:

(a) Two high titre anti-cholera sera agglutinated heated *Yersinia* antigen to only a low titre of 40–80 (Table 3) which was absorbed by heated Inaba, Ogawa and also *Yersinia* (Table 2) but not by live *Yersinia* antigen (Table 3). All antisera, including those produced by injecting live *Yersinia* antigen, failed to agglutinate live suspension of *Yersinia* probably because of the presence of a surface antigen (Table 1).

(b) None of the four anti-yersinia sera reacted in the agglutination test with heated *V. cholerae* antigen: agglutinin titre against live Inaba antigen was low, 40 in three sera and 160 in one (Table 1).

(c) As mentioned earlier, *Yersinia* and cholera antisera could not be tested for bactericidal activity against *Y. enterocolitica* to examine whether *V. cholerae* produced bactericidal antibody against *Yersinia* in the same way that *Yersinia* induced vibriocidal antibody.

In spite of these limitations, it could be shown by the absorption studies that while the Inaba vibriocidal antibody in anti-yersinia rabbit sera could be removed by *V. cholerae* Inaba (but not Ogawa) as well as by *Yersinia*, this same activity could not be absorbed from the anti-cholera sera by *Yersinia* cells. It is also interesting to note that Inaba antigen removed the vibriocidal activity from anti-yersinia sera without affecting the *Yersinia* agglutinating activity of the same

sera, indicating different antigenic determinants in *Yersinia* for these two different types of antibodies. When anti-yersinia sera were absorbed with *Yersinia* antigen, both the vibriocidal and *Yersinia* agglutinating antibodies were removed (Table 2). These observations were also confirmed by absorption of five human sera (Table 5), although complete cross absorption studies of these sera could not be done because of the limited amount of sera available.

The results of the present study also indicate that the antigen responsible for vibriocidal antibody formation in *Yersinia enterocolitica* serotype IX is heat-stable as in the case of *V. cholerae*, though the heated, like the live *Yersinia* antigen was ineffective in absorbing the vibriocidal activity in the anti-cholera sera. This type of non-reciprocal relationship was also described between the O antigens of *Sh. dysenteriae* 4 and *E. coli* O group 88 and between the O antigens of *Sh. dysenteriae* 1 and *E. coli* O group 120 by Ewing (1953) on the basis of agglutination reaction; no satisfactory explanation was offered for this phenomenon.

Heterophile antibodies might have explained these peculiar serological reactions, and all antisera prepared in rabbits were tested and found negative for agglutinin against sheep red cells by the technique of Davidsohn & Walker (1935).

In view of the fact that the cross-reacting antibody produced by *Yersinia* was mostly vibriocidal in nature against Inaba and that Inaba agglutinating antibody was found in 4 out of 9 and Ogawa agglutinating antibody in 1 out of 9 sera from human *Yersinia* infection, it appears that the somatic agglutinin against *V. cholerae* is less liable to non-specific stimulation as far as *Y. enterocolitica* serotype IX is concerned.

The present observations indicate that the results of sero-epidemiological surveys and immunological studies for cholera based on vibriocidal antibody titration should be interpreted with caution, particularly in areas where organisms serologically cross-reacting with *V. cholerae*, including *Y. enterocolitica* type IX, are prevalent.

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